Deletion of Protein Tyrosine Phosphatase Nonreceptor Type 2 in Intestinal Epithelial Cells Results in Upregulation of the Related Phosphatase Protein Tyrosine Phosphatase Nonreceptor Type 23

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Abstract: Background/Aims Knockdown of protein tyrosine phosphatase nonreceptor type 2 (PTPN2) exaggerates IFN- -induced intestinal barrier defects, but mice constitutively lacking PTPN2 in epithelial cells (PTPN2xVilCre mice) do not show changes in epithelial function or enhanced susceptibility to experimental colitis. Here, we investigated whether PTPN2 modulates the expression of related tyrosine phosphatases. Methods PTPN2 knockdown in HT-29 cells was induced using siRNA constructs. Acute colitis in PTPN2xVilCre mice was induced by 2% dextran sulfate sodium (DSS) in drinking water for 7 days. Colitis-associated tumors were induced by injection of azoxymethane prior to treatment with DSS for 3 consecutive cycles. Results In HT-29 cells, PTPN2 depletion resulted in enhanced mRNA expression of PTPN11 and PTPN23 and in parallel to upregulation of IL-18 mRNA upon treatment with TNF for 24 h. DSS treatment of PTPN2-deficient mice resulted in a strong induction of Ptpn23 mRNA in colon tissue in vivo. In the tumor model, Ptpn23 mRNA was again clearly upregulated in nontumor tissue from PTPN2-deficient mice; however, this was not observed in tumor tissue. Conclusions Our experiments show that PTPN23 function might, at least partially, compensate lack of PTPN2 in epithelial cells. Upregulation of PTPN23 might therefore crucially contribute to the lack of a colitis phenotype in PTPN2-VilCre mice.

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Deletion of PTPN2 in intestinal epithelial cells results in up-regulation of the related phosphatase PTPN23

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Short title: Enhanced PTPN23 expression upon deletion of PTPN2

Abbreviations: AOM: Azoxymethane; CRC: colorectal carcinoma; DSS: dextran sodium sulfate; IBD: Inflammatory Bowel Disease; IEC: intestinal epithelial cell; PTPN: protein tyrosine phosphatase non-receptor type

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Abstract

Background/Aims: Knock-down of protein tyrosine phosphatase non-receptor type 2 (*PTPN2*), exaggerates IFN-γ-induced intestinal barrier defects, but mice constitutively lacking *PTPN2* in epithelial cells (PTPN2xVilCre mice) do not show changes in epithelial function or enhanced susceptibility to experimental colitis. Here, we investigated whether PTPN2 modulates the expression of related tyrosine phosphatases.

Methods: PTPN2 knock-down in HT-29 cells was induced using siRNA constructs. Acute colitis in PTPN2xVilCre mice was induced by 2% DSS in drinking water for 7 days. Colitis-associated tumours were induced by injection of azoxymethane prior to treatment with DSS for 3 consecutive cycles.

Results: In HT-29 cells, *PTPN2*-depletion resulted in enhanced mRNA expression of *PTPN11* and *PTPN23* and in parallel to upregulation of *IL18* mRNA upon treatment with TNF for 24h. DSS treatment of PTPN2-deficient mice resulted in a strong induction of *Ptpn23* mRNA in in colon tissue *in vivo*. In the tumour model, *Ptpn23* mRNA was again clearly upregulated in non-tumour tissue from PTPN2-deficient mice, however, this was not observed in tumour tissue.

Conclusions: Our experiments show that PTPN23 function might, at least partially, compensate lack of PTPN2 in epithelial cells. Up-regulation of PTPN23 might therefore crucially contribute to the lack of a colitis phenotype in PTPN2-VilCre mice.

Key words: tyrosine phosphatases, HD-PTP; TC-PTP; intestinal inflammation; intestinal epithelial cells

1. Introduction

Inflammatory bowel disease (IBD) is characterized by a chronic, relapsing and remitting inflammation of the gastrointestinal tract. Patients suffer from abdominal pain, diarrhea, and extra-intestinal manifestations, including skin rashes, uveitis, and arthritis are common complications. Further, patients with longstanding disease course might be at a higher risk of developing both, gastrointestinal as well as extra-intestinal malignancies, including skin cancer, lymphoma, and colorectal cancer (CRC) [1]. The increased risk to develop CRC is primarily due to the fact that chronic inflammation and recurrent epithelial defects might promote aberrant proliferation and malignant transformation of intestinal epithelial cells [2].

Although the exact etiopathogenesis of IBD has not been completely understood, it is commonly accepted that genetic, immunological, environmental and bacterial factors play a key role in the development of chronic intestinal inflammation [3]. Genome-wide association studies (GWAS) identified 240 genetic loci that affect the susceptibility to develop IBD, including protein tyrosine phosphatase non-receptor type 2 (PTPN2, also referred to as T cell protein tyrosine phosphatase (TC-PTP)) [4-6]. Variants in the gene locus encoding PTPN2 are associated with both, CD and UC [7]. PTPN2 is a member of the protein tyrosine phosphatase (PTPs) family, which, together with protein tyrosine kinases (PTKs), are responsible for regulating the phosphorylation status of intracellular proteins [8]. Phosphorylation and de-phosphorylation of protein tyrosine residues are essential mechanisms for activating or de-activating intracellular signaling cascades.

PTPN2 is expressed in all tissues of the body, and in humans two splicing variants with slightly different subcellular localization patterns have been documented [9]. Since PTPN2 exerts obviously important anti-inflammatory functions, genetic deletion of PTPN2 in mice leads to death not later than 5 weeks after birth as a result of systemic inflammation with signs of severe colitis [10].

Intestinal epithelial cells (IECs) form a tight intestinal epithelial barrier to prevent food particles and intestinal bacteria from unlimited access to the host [11]. In IBD patients, defects in the epithelial barrier and increased intestinal permeability have been well documented [12,13]. An important cytokine that modulates intestinal permeability is IFN-y [14]. Of note, IFN-y also stimulates PTPN2

mRNA and protein expression, and PTPN2 in turn negatively regulates IFN-γ induced signaling cascades and dampens IFN-γ-induced barrier defects [15]. Elevated *PTPN2* mRNA and protein levels are found in colonic tissue samples of CD patients with acute inflammation, but not in samples of CD patients in remission [16]. PTPN2 knockdown in cell culture experiments exaggerates IFN-γ-induced epithelial barrier permeability, and promotes pro-inflammatory cytokine secretion [15]. These *in vitro* findings suggest that PTPN2 is important for the preservation of intestinal barrier functions, especially upon inflammatory insults. However, *in vivo* experiments from Kasper *et al.* showed that mice that specifically lack PTPN2 in IECs do not show enhanced disease severity upon induction of acute or chronic dextran sodium sulfate (DSS)-mediated colitis. Of note, those mice exhibit increased IEC proliferation and faster intestinal wound healing [17].

These rather unexpected findings led us to the hypothesis that in mice lacking PTPN2 in the intestinal epithelium, upregulated expression of other tyrosine phosphatases might compensate for the loss of PTPN2. Therefore, the aim of the here presented work was to investigate whether loss of PTPN2 in IECs results in up-regulation of non-receptor protein tyrosine phosphatases that de-phosphorylate similar target molecules as PTPN2, which might compensate for PTPN2 deficiency.

2. Material and Methods

2.1. Cell culture and siRNA transfections

HT-29 cells were obtained from DSMZ (Braunschweig, Germany) and cultured as described previously [17] in culture medium (DMEM; Thermo Fisher Scientific, Waltham, MA), supplemented with 1% non-essential amino acids (Thermo Fisher Scientific) and 10% FCS. For experiments, 1 x 10^6 cells were seeded in 12-well plates. For siRNA transfections, medium was changed to serum free DMEM, and 50pmol of three different PTPN2-specific siRNA constructs, or 50 pmol of three different non-targeting control siRNA constructs were mixed with RNAiMAX transfection reagent (Thermo Fisher Scientific) and applied to the cells. After 12h the medium was changed for normal culture medium. 24 h after the transfection, cells were treated with 100μg/ml IFN-y, or 100μg/ml TNF and RNA was harvested 24h later.

2.2. Mice

C57/Bl6 mice with a floxed PTPN2 gene (PTPN2fl/fl mice, termed wild-type throughout the manuscript) and PTPN2fl/fl mice expressing Cre-recombinase under the Villin promoter (PTPN2xVilCre mice) that lack PTPN2 specifically in intestinal epithelial cells [17] were used for all studies. Acute colitis was induced via administration of 2.5% DSS in the drinking water as described by Kasper et al. [17]. To induce colitis-associated tumours, mice were treated for 3 consecutive cycles consisting of 7 days with 1.5% DSS followed by 10 days recovery phase, each. At the first day of each cycle, mice were injected with 10mg/kg body weight azoxymethane (AOM). All animal experiments were performed according to local animal welfare legislation and were approved by the Veterinary Office of the Canton Zurich (ZH-255/2014).

2.3. RNA Isolation

Tissue samples were frozen in liquid nitrogen and stored in -80°C until RNA isolation. Cells were washed two times in ice cold PBS, lysed in 350 µl RLT buffer and homogenized using a 26G needle. Colon pieces were mechanically disrupted in 350 µl RLT buffer and Dithiothreitol (Qiagen, Düsseldorf, Germany) using the gentleMACS tissue disrupter according to the manufacturer's instructions. To

isolate total RNA, the RNeasy Plus Mini Kit (Qiagen) was used according to the manufacturer's instructions. RNA concentration was measured with a spectrophotometer NanoDrop ND1000 (NanoDrop Technologies, Wilmington, USA) by absorbance at 260 nm.

2.4. Reverse transcription and real-time polymerase chain reaction (RT-PCR)

A total of 1ug RNA was transcribed to complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368813). Real-time polymerase chain reaction (PCR) was performed using FAST qPCR MasterMix for Taqman Assays (Thermo Fisher Scientific) on a Fast HT7900 Real-Time PCR system using SDS Software (Thermo Fisher Scientific) with the following amplification steps: Initial enzyme activation for 5 min at 95°C, followed by 45 cycles of denaturing for 15 sec at 95°C and annealing/extending for 1 min at 60°C, each. Measurements were performed in triplicates using mouse β-actin as endogenous control. Results were analyzed using the $\Delta\Delta C_T$ method.

2.5. Western blotting

For Western blot analyses, cells were washed two times in PBS prior to lysis in M-PER buffer (Thermo Fisher Scientific) according to the manufacturer's instructions. After 30 min. lysates were centrifuged for 10 min at 17'000 x g and protein-containing supernatants collected into fresh tubes. Aliquots containing equal amounts of protein were loaded on 10% polyacrylamide gels and proteins separated for 1 h prior to blotting onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with 3% milk, 1% BSA in Tris-buffered saline containing 1% Tween (TBST) prior to incubation with anti-HD-PTP (1:100, Atlas antibodies, Stockholm, Sweden), anti PTPN2 (1:500, Millipore-Merck, Burlington, MA), anti-IL-18 (1:500; R&D Systems, Minneapolis, MN) or anti-b-actin (1:10'000; Millipore-Merck) antibodies over night. After washing three times in TBST, membranes were incubated with HRP-coupled secondary antibody (1:3'000; Santa Cruz Biotechnologies, Dallas, TX) for 1.5 h and immunoreactive proteins detected using an ELC substrate (Witec AG, Sursee, Switzerland).

2.6. Enzyme-linked Immunosorbent Assay (ELISA)

Anti-human and anti mouse IL-18 ELISA kits were purchased from R&D systems and 100 µl cell culture supernatant or 50 µl serum used for detection of IL-18 according to the manufacturer's instructions.

2.7. Immunhistochemistry (IHC)

IHC was performed on formalin-fixed, paraffin-embedded tissue specimen using a peroxidase-based method with diaminobenzidine (DAB) chromogen. Tissue samples were incubated with Histoclear (Chemie-Brunschwig, Basel, Switzerland) and descending concentrations of ethanol. Antigen retrieval was performed using citrate buffer, pH 6.0 (DAKO, Glostrup, Denmark) for 30 min at 98 °C. Endogenous peroxidases were deleted by incubation with 0.9 % hydrogen peroxide for 15 min at room temperature (RT) and blocking was performed using 3 % bovine serum albumin (BSA) for 2 h. Sections were stained over night at 4°C with an anti-mouse PTPN23 antibody (Novus biologicals, 1:50 dilution). HRP-labeled secondary anti-rabbit IgG-antibody (Vector laboratories, Peterborough, UK) was applied for 1 h at RT and antibody binding visualized by a Liquid DAB+ Substrate Chromogen System (Vector laboratories). Then samples were counterstained with hematoxylin, dehydrated in ascending concentrated ethanol and Histoclear solutions and finally mounted. Microscopic assessment was done using an AxioCam HRc (Zeiss, Jena, Germany) on a Zeiss Axio Imager.Z2 microscope (Zeiss) with AxioVision Release 4.8.2 software (Zeiss).

2.8. Statistics

All statistical analyses were performed using GraphPad Prism 5 Software (GraphPad Software Inc., La Jolla, USA). P-values below 0.05 were considered significant.

3. Results

3.1. PTPN2 controls the expression of *PTPN1*, *PTPN9* and *PTPN23* in HT-29 cells.

We first investigated whether loss of PTPN2 in IECs might affect the expression of other tyrosine phosphatases with similar targets to PTPN2 or with reported functions in intestinal inflammation, namely PTPN1 (also known as PTP1B), PTPN9, PTPN11, PTPN22 and PTPN23. To examine whether their mRNA expression is altered upon deletion of *PTPN2* in an IEC line, we treated HT-29 colon carcinoma cells with *PTPN2*-specific small interfering (si) RNA or non-targeting control siRNA constructs. Cells were then treated with 100µg/ml IFN-y or 100 µg/ml TNF for 24 h. As expected, mRNA expression of *PTPN2* was clearly reduced upon *PTPN2*-siRNA transfection showing that only 40-50% of the initial *PTPN2* mRNA expression was still retained (figure 1A).

We found that TNF-, but not IFN-y-treatment slightly enhanced the expression of *PTPN1* mRNA (figure 1B), while loss of PTPN2 resulted in lower basal *PTPN1* mRNA expression, and its induction was significantly decreased upon TNF-treatment when compared to PTPN2 competent cells ($p < 0.05$). Of note, without treatment, *PTPN9* mRNA expression was slightly, but significantly enhanced upon knockdown of PTPN2 ($p < 0.01$, figure 1C). TNF-treatment resulted in significantly reduced mRNA expression of *PTPN9* in PTPN2-competent, as well as in PTPN2-knockdown cells compared to their respective controls. *PTPN2* knockdown resulted in higher *PTPN9* mRNA expression already in untreated controls as well as in response to treatment with TNF. *PTPN11* mRNA expression was elevated in untreated PTPN2-knockdown cells when compared to untreated PTPN2-competent cells and TNF stimulation even further enhanced *PTPN11* levels ($p < 0.001$, figure 1D), while IFN-y decreased *PTPN11* mRNA levels in PTPN2-deficient cells. Further, IFN-y-treatment promoted *PTPN22* mRNA expression in PTPN2-deficient IECs ($p < 0.05$, figure 1 E), whereas *PTPN22* mRNA expression was not affected by TNF-stimulation ($p < 0.001$).

Of note, basal levels of *PTPN23* mRNA were significantly higher in PTPN2-knockdown cells when compared to PTPN2 competent HT-29 cells. This effect was clearly enhanced upon TNF-treatment ($p < 0.001$, figure 1F). TNF also induced PTPN23 mRNA levels in PTPN2 competent cells. Up-regulation of HD-PTP (the protein product of *PTPN23*) was confirmed by Western blotting, where we also found clearly enhanced HD-PTP levels upon treatment with TNF in PTPN2-competent cells, and increased

basal levels of HD-PTP in PTPN2-deficient cells. Of note the TNF-induced increase of HD-PTP was further potentiated in PTPN2-deficient cells, confirming our findings on mRNA level (figure 1G).

3.2. PTPN2 regulates the expression of pro-inflammatory cytokines in HT29 cells.

Having demonstrated that PTPN2 is involved in the regulation of the expression of mainly PTPN11 and PTPN23 in HT29 IEC, we next analyzed the mRNA expression of pro-inflammatory cytokines. TNF stimulated *IL-1b* expression in both, PTPN2-competent and PTPN2-siRNA treated HT29 cells, but this effect was clearly less pronounced in PTPN2-siRNA transfected cells ($p < 0.001$, figure 2A). *IL-18* mRNA expression on the other hand, was also upregulated upon TNF-stimulation in HT29 cells, and this effect was further enhanced in PTPN2-siRNA treated cells ($p < 0.001$, figure 2B). Even without treatment, PTPN2-deficient cells showed highly increased *IL-18* mRNA expression which was clearly reduced in response to IFN-y-stimulation ($p < 0.01$). Increased IL-18 levels were also confirmed in Western blot analyses (Figure 2C) and IL-18 ELISA (Figure 2D).

In addition, TNF stimulation resulted in highly increased mRNA expression of *IL-8* mRNA in both, PTPN2-deficient and control cells (figure 2E). However, and in line with previous reports, PTPN2-deficient cells expressed less *IL-8* mRNA in response to TNF when compared to control cells ($p < 0.001$). Since IFN-y-treatment had little or no effect in the so far analyzed genes, we confirmed response to IFN-y via analysis of mRNA levels of intercellular adhesion molecule-1 (ICAM-1). *ICAM-1* mRNA levels were highly increased upon IFN-y-stimulation in PTPN2-competent and even more in PTPN2-deficient IECs ($p < 0.001$, figure 2F). This was also true for TNF stimulation ($p < 0.001$).

3.3. PTPN23 mRNA expression is increased in colon lysates from DSS-treated PTPN2xVilCre mice

To further address the hypothesis that loss of PTPN2 might be compensated by altered expression of other tyrosine phosphatases, we next measured mRNA expression of *Ptpn2*, *Ptpn1*, *Ptpn9*, *Ptpn11*, *Ptpn22*, and *Ptpn23* in colon tissue samples of wildtype (wt) mice and mice lacking PTPN2 specifically in intestinal epithelial cells (PTPN2xVilCre mice). The mice had either been left untreated or treated with 2% dextran sulphate sodium (DSS) in the drinking water for seven days to induce an acute

colitis[17]. As already described in Kasper *et al* this treatment resulted in similar intestinal inflammation in wt and PTPN2-VilCre mice [17].

As depicted in figure 3, mRNA expression of *Ptpn2* was significantly reduced in colon pieces from PTPN2xVilCre mice, and loss of PTPN2 also resulted in a marked reduction of *Ptpn1* mRNA levels (figure 3A+B). On the other hand, we did not observe any significant changes in *Ptpn9* and *Ptpn11* mRNA expression in PTPN2xVilCre mice when compared to wt animals (figure 3C+D). Whereas DSS-treated wt animals showed slightly enhanced *Ptpn22* mRNA expression when compared to their control littermates without DSS, its expression was significantly decreased in PTPN2xVilCre mice that were treated with DSS ($p < 0.01$, figure 3E).

Of note, while *Ptpn23* mRNA was not altered in PTPN2xVilCre mice without induction of colitis, there was a clearly up-regulation of *Ptpn23* upon DSS treatment in PTPN2-xVilCre mice ($p < 0.01$, figure 3F). Up- regulation of PTPN23 in PTPN2-VilCre mice was further confirmed in immunohistochemical (IHC) staining of distal colon sections (Figure 3G).

We have previously shown that loss of PTPN2 promotes activation of inflammasomes [18], and subsequent secretion of IL-1β and IL-18. Therefore, we also addressed whether loss of PTPN2 in epithelial cells affects expression of *Il1b* and *Il18* mRNA. While *Il1b* mRNA expression level was not altered in all four groups, *Il18* mRNA expression showed a trend towards a reduction in DSS treated wt and PTPN2xVilCre mice compared to their corresponding controls (figure 3H+I), possibly due to a negative feedback regulation. This hypothesis was confirmed by addressing IL-18 levels in serum by ELISA, where we found increased IL-18 protein levels in DSS treated WT mice when compared to water controls. Of note, this increase was further enhanced in DSS-treated PTPN2-VilCre mice (Figure 3J). This is in line with our previous finding that the regulation of IL-1β and IL-18 via PTPN2 is mediated via changes in protein activation, and not via changes in mRNA levels [18].

3.4. mRNA expression level in colon lysates from AOM-DSS treated PTPN2xVilCre mice

In a next step, we aimed to address whether loss of PTPN2 also affects expression of other phosphatases in a model of colitis-associated tumour formation. For this aim, wt mice and PTPN2xVilCre littermates were subjected to a commonly used mouse model for colorectal carcinoma, namely the AOM/DSS induced colon tumour model [19]. This treatment resulted in colon inflammation

and tumor development as previously described in Spalinger *et al* [18]. In this experiment, colon tissue pieces from non-tumour tissue and from tumour tissue have been collected for mRNA analysis and we here investigated the expression levels of *Ptpn2*, *Ptpn1*, *Ptpn9*, *Ptpn11*, *Ptpn22*, *Ptpn23*, *Il1b* and *Il18* mRNA.

Ptpn2 mRNA expression was decreased in PTPN2xVilCre mice, but not affected by DSS/AOM treatment in tumour or non-tumour tissue (figure 4A). As shown in figure 4B, *Ptpn1* mRNA expression tended to be higher in non-tumour tissue from mice treated with AOM/DSS than in control littermates, although this effect was not statistically significant. Regardless of the genotype, there was significantly more *Ptpn1* expressed in tumour tissue than in colon tissue from healthy wt mice ($p < 0.01$, figure 4B) – this was in contrast to our findings obtained using acute DSS colitis model, where *Ptpn1* expression was markedly decreased upon deletion of *PTPN2*. In contrast to our cell culture experiment, but in line with results from the DSS acute colitis, *Ptpn9* expression was not affected by AOM/DSS treatment and did not show an altered mRNA expression profile. Likewise, *Ptpn11* mRNA levels were not affected by treatment or genotype (figure 4C+D).

Ptpn22 mRNA expression was increased in non-tumour colon tissue from AOM/DSS treated PTPN2xVilCre mice compared to healthy PTPN2xVilCre ($p < 0.05$) and wt control mice ($p < 0.01$, figure 4E). Upon AOM/DSS treatment, *Ptpn22* expression was significantly decreased in tumour tissue from wt mice, when compared to non-tumour tissue from the same mice ($p < 0.05$, figure 4E). Although there seems also to be a decrease of *Ptpn22* expression in tumour tissue from PTPN2xVilCre mice, this reduction was far less pronounced, and tumour tissue from PTPN2xVilCre mice tended to express more *Ptpn22* than wt tumour tissue (figure 4E).

In the colon tumour mouse model, we observed that *Ptpn23* mRNA expression was clearly increased in colonic non-tumour tissue derived from PTPN2xVilinCre mice after AOM/DSS treatment compared to wt control mice ($p < 0.01$, figure 4F) and wt mice treated with AOM/DSS ($p < 0.05$). Interestingly, *Ptpn23* mRNA expression was not altered in tumour tissue of neither PTPN2 wt nor PTPN2xVilCre mice when compared to the respective untreated control mice. mRNA expression data was then confirmed by IHC, where we found increased HD-PTP expression in PTPN2-VilCre mice in general, and a slightly more pronounced increase in tumor tissue from PTPN2-VilCre mice (Figure 5).

When analyzing the expression of *Il1b* and *Il18,* we observed that in general, *Il1b* mRNA was upregulated upon AOM/DSS treatment (figure 6A+B). The strongest increase in expression was found in tumour tissue of wt mice, followed by tumour tissue of PTPN2-VilCre mice. In contrast to *Il1b*, *Il18* mRNA expression was reduced in wt mice treated with AOM/DSS ($p < 0.01$) and in tumour tissue from wt ($p < 0.01$) and PTPN2xVilCre mice ($p < 0.05$). Surprisingly, *Il18* mRNA expression was highest in wt control mice when compared to all other groups, which, again might result from a negative feedback regulation. This hypothesis was confirmed using ELISA for IL-18, where we found a clear trend towards increased serum levels in AOM/DSS treated PTPN2-VilCre mice (Figure 6C).

4. Discussion

In this study, we investigated how loss of PTPN2 in IECs modulates the expression of related tyrosine phosphatases in HT-29 cells, colon lysates and tumour tissue of PTPN2xVilCre mice. Our results showed that *in vivo*, loss of PTPN2 in intestinal epithelial cells mainly affected the expression of *Ptpn23* mRNA, while *Ptpn9* and *Ptpn11* were not changed in a consistent manner among the different experimental settings. This was supported by our HT29 cell line studies, where we identified as a most striking finding a regulatory loop between PTPN2 and PTPN23 in IECs (Figure 7).

In the *in vivo* setting we found that among the studied tyrosine phosphatases, only PTPN23 was more expressed in tissue of PTPN2xVilCre mice compared to their wt littermates. This result is consistent with our finding in the *in vitro* experiments, where *PTPN23* mRNA expression was also increased in all three groups of HT29-PTPN2-knockdown cells compared to the respective control groups. It has been shown that *PTPN23* is essential during embryonic development and its expression in adult mice is limited to the epithelium of multiple organs including the epithelium of the stomach, small intestine and colon [20]. *PTPN23* encodes for the protein HD-PTP, which controls cell migration and endocytosis [21]. The role of *PTPN23* in IECs in the intestine has not been studied yet. However, our recent unpublished findings indicate that PTPN23/HD-PTP might control proliferation and migration of IECs. Interestingly, *PTPN23* expression was only increased in non-tumour tissue from PTPN2xVilCre mice treated with AOM/DSS but not in the tumour tissue itself. This indicates that in the course of tumour formation and progression, *PTPN23* upregulation seems to be lost and therefore also its compensatory function. This is in line with several studies that show that loss of *PTPN23* promotes the development of breast cancer and that hemizygous loss of *PTPN23* facilitates spontaneous tumourgenesis [22,23].

A very interesting observation is that in PTPN2-deficient cells, the mRNA expression pattern of IL-18 follows the expression pattern of PTPN23. This is in contrast to *Il1b* mRNA levels. Those findings suggest that in absence of PTPN2, PTPN23 might play a critical role for the regulation of IL-18 expression and it might be interesting to investigate whether PTPN23 might be involved in the regulation of IL-18 protein expression and activation as well.

In addition to PTPN23 upregulation, *PTPN22* mRNA expression was reduced in PTPN2xVilCre mice upon DSS treatment. We have previously shown that PTPN22 is reduced in the intestine of IBD patients and that it plays a protective role during intestinal inflammation [24]. Further, PTPN22 deficient mice are more affected by DSS-induced colitis [25-27]. One possible explanation for reduced PTPN22 expression might be the fact that TNF and IL-1β supress *PTPN22* expression [28]. The findings that PTPN22 was clearly reduced in the tumour tissue compared to the non-tumour tissue in wild-type and PTPN2xVilCre mice suggests that PTPN22 might also exert somehow a tumour suppressor function in the colon regardless of PTPN2.

Of note, we found reduced *PTPN1* mRNA expression in PTPN2xVilCre mice in the acute DSS experiment, regardless whether the mice were treated with DSS or not, while this was not the case in the AOM-DSS experiment. This seems contradictory at first, but might be explained by the fact that the mice are several weeks older at the end of the AOM-DSS tumour induction. We showed that *in vitro, PTPN1* expression is clearly regulated upon presence of inflammatory cytokines. Low grade inflammatory processes might therefore account for a lack of genotype-specific differences in older mice. PTPN1 is closely related to PTPN2, sharing approximately 70% of their catalytic domain sequence [29]. Since PTPN1 is a negative regulator of JAK/STAT-mediated signalling regulating myeloid-derived suppressor cells, PTPN1 null mice are more resistant to DSS-induced colitis due to expansion of protecting myeloid-derived suppressor cells that modulate cytokine secretion [30]. Therefore, the marked reduction of PTPN1 in our acute experiments might also contribute to the fact that PTPN2xVilCre mice do not suffer from pronounced acute colitis.

We observed that *PTPN9* mRNA was highest in untreated PTPN2-deficient HT-29 cells. Its expression decreased strongly upon stimulation with the pro-inflammatory cytokines IFN-y and TNF. However, the expression of *PTPN9* mRNA was not changed in PTPN2xVilCre mice during induction of colitis, and it is well possible that other inflammatory cytokines, such as IL-6 or IL-1 might counteract the effects of TNF/IFN-y in the *in vivo* situation. Hence, further studies would be necessary to fully understand the regulation of *PTPN9* in the (inflamed) intestine.

Interestingly, we found increased expression of *PTPN11* in PTPN2-deficient HT29 cells upon TNF-stimulation but not upon IFN-y treatment. This might be explained by the regulatory function of PTPN2

on IFN-γ-induced signalling cascades [31]. Several studies proposed that PTPN11 is a protective factor against gut inflammation [28,32] and its upregulation in IECs might represent a compensatory effect for the loss of PTPN2. Nevertheless, we did not observe significant differences in *PTPN11* mRNA expression in colon lysates from PTPN2xVilCre mice upon colitis induction, leading to the conclusion that this phosphatase might play a subordinate role to compensate for the loss of PTPN2 *in vivo*. Further, in the *in vivo* situation, only IEC lack PTPN2, while all other cells express normal PTPN2 levels. In our study, we measured RNA in whole colon tissue, which includes IEC, as well as myeloid and lymphoid immune cells. Although our results show that many of the *in vitro* effects are mirrored in the whole-tissue studies, the effect on PTPN11 expression observed in the samples from the *in vivo* experiment might reflect the effects resulting from non-IEC cells. Given the complex interaction between phosphatases and various intracellular signalling molecules, network analyses might be an interesting further direction to more completely understand how loss of PTPN2 in IEC affects cellular signalling pathways as a whole, as well as possible interaction between different cell types present in the intestine.

It should be considered that in our cell culture experiment, knockdown of *PTPN2* was not complete, hence the effects in the *in vitro* experiments might be less pronounced than from a complete knockout, and might more closely reflect the situation observed in patients where presence of the variants results in reduced PTPN2 function but not its complete loss.

Taken together, our results demonstrate that depletion of PTPN2 in intestinal epithelial cells affects the expression of other tyrosine phosphatases, which might contribute to the fact that mice lacking PTPN2 specifically in IECs do not develop pronounced colitis. Most strikingly, there was a marked, and consistent upregulation of PTPN23 in PTPN2 deficient cells and in colon tissue from PTPN2xVilCre mice, indicating that *in vivo*, this phosphatase might be the most relevant to compensate for the lack of PTPN2.

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6. Figure Legends

Figure 1. Deletion of PTPN2 in HT-29 cells results in enhanced PTPN9, PTPN11 and PTPN23 expression.

HT-29 cells were treated with non-targeting control siRNA or with PTPN2-specific siRNA and activated with 100 ng/ml IFN-y or 100 ul/ml TNF for 24h as indicated. Cell lysates were analysed for mRNA expression of A) *PTPN2,* B) *PTPN1,* C) *PTPN9,* D) *PTPN11,* E) *PTPN22,* and F) *PTPN23*. Data were normalized to cells treated with control siRNA without activation. Asterisks denote significant differences * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Newman Keul's test; Ctr, Control; Actb, actin beta.

Figure 2. Deletion of PTPN2 in HT-29 cells affects mRNA expression of pro-inflammatory cytokines. HT-29 cells were treated with non-targeting siRNA or with PTPN2-specific siRNA and activated with 100 ng/ml IFN-y or 100 ul/ml TNF for 24h as indicated. Cell lysates were analysed for mRNA expression of A) *IL1B*, and B) *IL18*. C) Protein levels of IL-18 were analysed by Western blot and ELISA. mRNA levels of E) *IL8,* and F) *ICAM1* were analysed by qPCR. Data were normalized to cells treated with control siRNA without activation. Asterisks denote significant differences * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Newman Keul's test; Ctr, Control; Actb, actin beta.

Figure 3. Colon lysates from DSS-treated PTPN2xVilCre mice show increased expression of PTPN23.

C57/Bl6 mice with a floxed *Ptpn2* gene (wt) and PTPN2fl/fl mice expressing Cre-recombinase under the Villin promoter that lack *Ptpn2* specifically in intestinal epithelial cells (PTPN2xVilCre mice) were treated with water or 2% DSS. Colon lysates of PTPN2xVilCre mice were analysed for mRNA expression of A) *Ptpn2,* B) *Ptpn1,* C) *Ptpn9,* D) *Ptpn11,* E) *Ptpn22,* and F) *Ptpn23* by RT-PCR. Data were normalized to Actb. G) Paraffin-embedded tissue from the terminal colon was stained for HD-PTP (brown), the protein product of *PTPN23*. mRNA expression of H) IL-1b and I) IL18 were analysed by RT-qPCR, and J) serum IL-18 levels detected by ELISA. Each symbol stands for one individual mouse and horizontal bars indicate the mean. Asterisks denote significant differences * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Newman Keul's test; wt, wildtype; DSS, dextran sulphate sodium; Actb, actin beta (housekeeping gene, mouse).

Figure 4. PTPN23 expression is enhanced in PTPN2xVilCre mice after AOM/DSS treatment but not increased in tumour tissue.

To induce colitis-associated tumours, PTPN2xVilCre mice were treated for 3 consecutive cycles consisting of 7 days with 1.5% DSS followed by 10 days recovery phase, each. At the first day of each cycle, 10mg/kg body weight Azoxymethane (AOM) was injected. Colon lysates and tumour pieces were analysed for mRNA expression of A) PTPN2, B) PTPN1, C) PTPN9, D) PTPN11, E) PTPN22, and F) PTPN23 by RT-PCR. Data were normalized to Actb. Asterisks denote significant differences * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Newman Keul's test; wt, wildtype; ko, knockout; AOM, azoxymethane; AOM T, azoxymethane treated group, tumour tissue.

Figure 5. PTPN23 protein expression is enhanced in PTPN2xVilCre mice after AOM/DSS treatment.

To induce colitis-associated tumours, PTPN2xVilCre mice were treated for 3 consecutive cycles consisting of 7 days with 1.5% DSS followed by 10 days recovery phase, each. At the first day of each cycle, 10mg/kg body weight Azoxymethane (AOM) was injected. Paraffin-embedded tissue from the terminal colon was stained for HD-PTP (brown), the protein product of *PTPN23*.

Figure 6. Altered expression of inflammatory cytokines in PTPN2xVilCre mice after AOM/DSS treatment. Mice were treated as in Figure 4 and colon lysates and tumour pieces analysed for A) IL-1b and B) IL-18 mRNA expression. Data were normalized to Actb. Asterisks denote significant differences * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Newman Keul's test; wt, wildtype; ko, knockout; AOM, azoxymethane; AOM T, azoxymethane treated group, tumour tissue.

Figure 7. Proposed mechanism how PTPN23 might counteracts the effect of PTPN2 deficiency. Deletion of PTPN2 promotes pro-inflammatory and pro-oncogenic signalling cascades, such as JAK/STAT signalling, EGFR signalling and IL-18 secretion. At the same time, it promotes the expression of PTPN23. PTPN23 in turn inhibits EGFR signalling and IL-18 induced effects, thus counteracting the effects of PTPN2 deficiency. Blue arrows: enhanced expression, red arrows with blunt ends: suppression. Dashed lines: proposed effects, solid lines: effects supported by literature.